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Z. Elizabeth Floyd
Louisiana State University

Jacqueline M. Stephens
Louisiana State University

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Interferon- γ -mediated Activation and Ubiquitin-Proteasome-dependent Degradation of PPAR γ in Adipocytes*

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Z. Elizabeth Floyd and Jacqueline M. Stephens‡

From the Department of Biological Sciences, Louisiana State University, Baton Rouge, Louisiana 70803

Interferon- γ (IFN γ) treatment of adipocytes results in a down-regulation of the peroxisome proliferator-activated receptor γ (PPAR γ). The decrease in PPAR γ expression is mediated by inhibition of PPAR γ synthesis and increased degradation of PPAR γ . In this study, we demonstrate that both PPAR γ 1 and PPAR γ 2 are targeted to the proteasome under basal conditions and that PPAR γ 1 is more labile than PPAR γ 2. The IFN γ -induced increase in PPAR γ turnover is blocked by proteasome inhibition and is accompanied by an increase in PPAR γ -polyubiquitin conjugates. In addition, IFN γ treatment results in the transcriptional activation of PPAR γ . Similar to ligand-dependent activation of PPAR γ , IFN γ -induced activation was greater in the phosphorylation-deficient S112A form of PPAR γ when compared with wild-type PPAR γ . Moreover, the inhibition of ERKs 1 and 2 with a MEK inhibitor, U1026, lead to an inhibition in the decay of PPAR γ proteins, indicating that serine phosphorylation influences the degradation of PPAR γ in fat cells. Our results also demonstrate that the proteasome-dependent degradation of PPAR γ does not require nuclear export. Taken together, these results indicate that PPAR γ is targeted to the ubiquitin-proteasome pathway for degradation under basal conditions and that IFN γ leads to an increased targeting of PPAR γ to the ubiquitin-proteasome system in a process that is affected by ERK-regulated serine phosphorylation of PPAR γ proteins.

PPAR γ ¹ is a member of the nuclear hormone receptor family, a group of transcription factors that are activated by small lipophilic ligands (1). PPAR γ exists as two isoforms, PPAR γ 1 and PPAR γ 2, which are produced by a combination of different promoters and alternative splicing (2). There is also a PPAR γ 3 gene that codes for a protein that is identical to PPAR γ 1 (3). PPAR γ 1 is predominantly expressed in fat cells but occurs in low levels in multiple tissues. PPAR γ 2 has an N-terminal extension of 30 amino acids and is very highly expressed in adipocytes (4, 5). Deletion of the PPAR γ gene in mice results in placental dysfunction and embryonic lethality (6, 7).

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‡ To whom correspondence should be addressed: Louisiana State University, Dept. of Biological Sciences, 508 Life Sciences Bldg., Baton Rouge, LA 70803. Tel.: 225-578-1749; Fax: 225-578-2597; E-mail: jsteph1@lsu.edu

¹ The abbreviations used are: PPAR γ , peroxisome proliferator-activated receptor γ ; TZD, thiazolidinedione; IFN γ , interferon- γ ; STAT, signal transducer and activator of transcription; HA, hemagglutinin; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; LMB, leptomycin B.

PPAR γ has been implicated in the regulation of systemic insulin sensitivity. This was first demonstrated when PPAR γ was shown to be a functional receptor for the synthetic antidiabetic thiazolidinediones (TZDs) (8). Thiazolidinediones are specific high affinity ligands for PPAR γ and the order of their receptor binding affinities *in vitro* mirrors their antihyperglycemic activity *in vivo* (9). Direct evidence for the association between PPAR γ and insulin sensitivity comes from genetic studies showing that mutations in the ligand-binding domain of PPAR γ are associated with severe insulin resistance. Although not obese, these patients developed type 2 diabetes as well as early onset hypertension (10). Also, insulin has been shown to acutely regulate the expression of PPAR γ in human adipocytes (11), and mice that only express one copy of the PPAR γ gene have been shown to be more sensitive to insulin (12). We have recently demonstrated that IFN γ results in a substantial loss of PPAR γ expression by regulating two cellular events: 1) targeting PPAR γ to the proteasome for degradation, and 2) inhibiting the synthesis of PPAR γ (13). Moreover, prolonged IFN γ treatment of 3T3-L1 adipocytes also results in the development of insulin resistance (13) and supports the hypothesis that PPAR γ is involved in conferring insulin sensitivity.

Interferon- γ (IFN γ) is a cytokine that is primarily known for its roles in immunological responses but has also been shown to affect fat metabolism and adipocyte gene expression. In adipocytes, IFN γ treatment results in a decrease of lipoprotein lipase (LPL) activity and increased lipolysis (14). In 3T3-F442 adipocytes, exposure to IFN γ results in a decreased expression of lipoprotein lipase and fatty acid synthase. Also, in various rodent preadipocyte cell lines, IFN γ inhibits the differentiation of preadipocytes (15–17). Acute IFN γ treatment of cultured and native rat adipocytes results in a dose- and time-dependent activation of STATs 1 and 3 (18). Moreover, there are studies (19–21) linking IFN γ and insulin resistance in humans. IFN γ has been implicated in the development of insulin resistance during viral infections (20), and IFN γ therapy of cancer patients has been associated with the development of hyperglycemia (21).

The ubiquitin-proteasome pathway is essential for the degradation of short lived proteins, the levels of which are regulated constitutively or in response to changes in the cellular environment (22, 23). Transcription factors and tumor suppressors are among the proteins regulated by the ubiquitin-proteasome pathway, and included in this group are members of the nuclear hormone receptor superfamily (24, 25). Ligand-dependent down-regulation by the ubiquitin-proteasome system has been demonstrated for several members of the nuclear hormone receptor family, including the estrogen (26, 27), progesterone (28), thyroid hormone (29), and aryl hydrocarbon receptors (30).

Substrates of the ubiquitin-proteasome system are targeted to the proteasome after covalent attachment of multiple ubiquitin molecules. Ubiquitin, a 76 amino acid protein, is initially

activated by E1, the ubiquitin-activating enzyme. Activated ubiquitin is then transferred to a ubiquitin-conjugating enzyme (E2), which generally shuttles ubiquitin to ubiquitin ligase (E3). E3 is bound to the targeted substrate and catalyzes the covalent attachment of ubiquitin to the substrate. Once the first ubiquitin is transferred to the substrate, a polyubiquitination chain is generated via a series of isopeptide linkages. The multiubiquitinated substrate protein is then degraded by the 26 S proteasome in an ATP-dependent manner (31).

Our recent studies (13) have shown that acute IFN γ treatment of 3T3-L1 adipocytes results in a repression of PPAR γ transcription that is independent of new protein synthesis. Yet, we also demonstrated that the half-life of PPAR γ proteins was shorter following IFN γ treatment. In the current investigation, we observed that proteasomal inhibitors attenuate the TZD- and IFN γ -induced decrease in PPAR γ expression. Moreover, we demonstrate that IFN γ treatment is associated with an increase in the formation of polyubiquitin-PPAR γ conjugates in 3T3-L1 adipocytes. Together, these data indicate that IFN γ signaling results in the increased targeting of PPAR γ to the ubiquitin-proteasome system in adipocytes. In addition, we have shown that like TZDs, IFN γ increases the transcriptional activity of PPAR γ . Also, the IFN γ -induced activation of a phosphorylation-deficient mutant of PPAR γ 2 (S112A) is substantially greater than the IFN γ activation of wild-type PPAR γ 2. Our results suggest that phosphorylation of PPAR γ 2 at Ser¹¹² contributes to the targeting of PPAR γ to the ubiquitin-proteasome pathway. Finally, these studies indicate that the IFN γ -mediated ubiquitin-proteasome-dependent degradation of PPAR γ occurs in the nucleus.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco's modified Eagle's medium (DMEM), Opti-MEM, and fetal bovine serum were purchased from Invitrogen. Calf serum was purchased from Sigma. Murine IFN γ was purchased from Roche Molecular Biochemicals. PPAR γ monoclonal (E-8, no. sc-7273) and polyclonal (H-100, no. sc-7196) antibodies, Mdm2 monoclonal (SMP14, no. sc-965) antibody, and a STAT 5A polyclonal (L-20, no. sc-1081) antibody were purchased from Santa Cruz Biotechnology. Monoclonal anti-ubiquitin (no. 13-1600) was purchased from Zymed Laboratories Inc. The proteasome inhibitors epoxomicin, lactacystin, and MG132 (N-carboxbenzoxyl-Leu-Leu-Leucinal) were purchased from Boston Biochemicals. A luciferase assay system, pSV- β -galactosidase control vector, and a β -galactosidase enzyme assay kit were purchased from Promega. FuGENE 6 was purchased from Roche Molecular Biochemicals. Darglitazone was kindly provided by Pfizer.

Constructs—The pSVSPORT plasmids encoding wild-type PPAR γ and the S112A PPAR γ mutant as well as DR-1 luciferase were the generous gift of Dr. Bruce Spiegelman (Dana Farber Cancer Institute). The HA-ubiquitin plasmid and leptomycin B (LMB) were kindly provided by Dr. Dirk Bohmann (European Molecular Biology Laboratories) and Dr. Minoru Yoshida (The University of Tokyo), respectively.

Cell Culture—Murine 3T3-L1 preadipocytes were plated and grown to 2-days postconfluence in DMEM with 10% calf serum. The medium was changed every 48 h. Cells were induced to differentiate by changing the medium to DMEM containing 10% fetal bovine serum and 0.5 mM 3-isobutyl-methylxanthine, 1 μ M dexamethasone, and 1.7 μ M insulin (MDI). After 48 h, this medium was replaced with DMEM supplemented with 10% fetal bovine serum, and the cells were maintained in this medium until used for experimentation. NIH 3T3 cells were grown in DMEM with 10% calf serum.

Preparation of Whole Cell Extracts—Cell monolayers were rinsed with phosphate-buffered saline (PBS) and harvested in a lysis buffer containing 10 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 0.5% Nonidet P-40, 1 μ M phenylmethylsulfonyl fluoride, 1 μ M pepstatin, 50 trypsin inhibitory milliunits of aprotinin, 10 μ M leupeptin, and 2 mM sodium vanadate. Samples were extracted on ice for 30 min prior to centrifugation at 10,000 $\times g$ for 15 min. The resulting supernatants were analyzed for protein content by BCA analysis (Pierce) according to the manufacturer's instructions and stored at -80°C .

Preparation of Nuclear/Cytosolic Extracts—Cell monolayers were rinsed with PBS and harvested in a nuclear homogenization buffer

(NHB) containing 20 mM Tris-Cl, pH 7.4, 10 mM NaCl and 3 mM MgCl₂. Nonidet P-40 was added to a final concentration of 0.15%, and the cells were homogenized with 16 strokes in a Dounce homogenizer. The resulting homogenate was centrifuged at 1500 rpm for 5 min, and the supernatant was saved as cytosolic extract. The nuclear pellet was twice resuspended in 0.5 volume of a nuclear homogenization buffer and centrifuged as before. The nuclear pellet was then resuspended in an extraction buffer containing 20 mM HEPES pH 7.9, 420 mM NaCl, 0.2 mM EDTA and 25% glycerol. Nuclei were extracted for 30 min on ice followed by incubation with 200 units of DNase I at room temperature for 15 min. Finally, the sample was centrifuged at 15,000 rpm for 10 min at 4°C . The resulting nuclear extract and the previously obtained cytosolic extract were analyzed for protein content by BCA analysis (Pierce) according to the manufacturer's instructions and stored at -80°C .

Gel Electrophoresis and Immunoblotting—Proteins were separated in 12% polyacrylamide (National Diagnostics) gels containing SDS according to Laemmli (32) and transferred to nitrocellulose (Bio-Rad) in 25 mM Tris, 192 mM glycine, and 20% methanol. Following transfer, the membrane was blocked in 4% milk overnight at 4°C . The immunoblots were visualized with horseradish peroxidase-conjugated secondary antibodies (Sigma) and enhanced chemiluminescence (Pierce).

Transient Transfection and Luciferase Assay—NIH 3T3 cells were grown to 60–70% confluence and transiently transfected with either wild-type PPAR γ 2 or PPAR γ 2 S112A. To measure PPAR γ activity, the cells were cotransfected with DR-1 luciferase and pSV- β -galactosidase to normalize for transfection efficiency. FuGENE 6 was used according to the manufacturer's instructions and a FuGENE 6 to DNA ratio of 3:2 was used in the transfections. Transient transfections were carried out in OptiMEM for 8 h. After 8 h, the media were replaced with DMEM supplemented with 10% calf serum, and the cells were incubated overnight. Twenty-four hours after transfection, the cells were treated with IFN γ (100 units/ml) or darglitazone (TZD) (2.5 μ M), and the cells were harvested 6 h later. Cell lysates were prepared and analyzed for luciferase activity and β -galactosidase activity according to the manufacturer's instructions (Promega). PPAR γ transcriptional activity was reported as the ratio of luciferase activity (relative light units) to β -galactosidase activity.

Ubiquitin Conjugation Assay—NIH 3T3 cells were transfected with 2 μ g of PPAR γ alone or in combination with 4 μ g of HA-ubiquitin per 100 mm plate using FuGENE 6 as described above. After 24 h, the cells were treated with 10 μ M MG132 for 2 h prior to the addition of IFN γ (100 units/ml). The cells were harvested after 15- and 30-min incubations and lysed on ice in PBS, pH 7.0, containing 1% Triton X-100, 10 mM N-ethylmaleimide, 1 mM phenylmethylsulfonyl fluoride, 1 μ M pepstatin and 10 μ M leupeptin. Immunoprecipitations were performed by incubation with a polyclonal anti-PPAR γ followed by incubation with protein A-Sepharose (RepliGen). PPAR γ -ubiquitin complexes were detected by Western blotting with an anti-HA antibody.

3T3-L1 adipocytes were serum-deprived overnight in OptiMEM, followed by incubation with 10 μ M MG132 for 2 h. At the end of 2 h, IFN γ (100 units/ml) was added, and the cells were harvested after 15- and 30-min incubations and lysed on ice in PBS containing 1% Triton X-100, 10 mM N-ethylmaleimide, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μ M pepstatin, and 10 μ M leupeptin. Immunoprecipitations were performed with a polyclonal anti-PPAR γ , and PPAR γ -ubiquitin complexes were detected by Western blotting using both anti-PPAR γ (monoclonal) and anti-ubiquitin antibodies.

PPAR γ Stability in Vivo—Experiments using 3T3-L1 adipocytes were carried out in the presence or absence of cycloheximide (5 μ M) to examine the effect of IFN γ on the half-life of PPAR γ proteins. The half-lives of PPAR γ 1 and PPAR γ 2 were calculated based on first order decay after quantitation of Western blot data using Un-Scan-It software (Silk Scientific, Inc). IFN γ was added at 100 units/ml and darglitazone was added at 2.5 μ M, where indicated. The adipocytes were incubated with one of three proteasome inhibitors (5 μ M lactacystin, 100 nM epoxomicin, or 10 μ M MG132) in experiments designed to assay proteasome targeting of PPAR γ . In these experiments, the cells were preincubated with the proteasome inhibitor for 15–30 min prior to adding the ligand or cycloheximide. A MAPK/ERK kinase (MEK) inhibitor, U0126 (5 μ M), was used to assay involvement of ERK1/2 in the turnover of PPAR γ , and the cells were preincubated with U0126 for 30–45 min. Leptomycin B (10 nM) was added as an inhibitor of CRM-1-dependent nuclear export (33). Cells were pretreated with leptomycin B for 0.5–1 h prior to the addition of ligand or cycloheximide. Vehicle control additions were performed with either Me₂SO (for proteasome inhibitors, TZDs, and U0126) or ethanol (for leptomycin B).

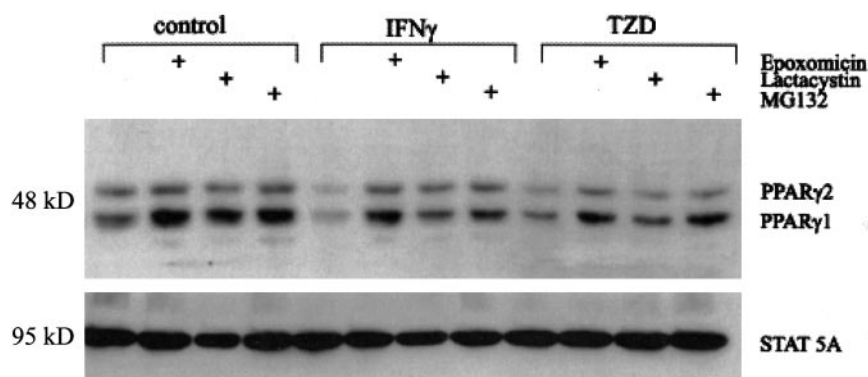


FIG. 1. PPAR γ is targeted to the proteasome under basal conditions and after IFN γ or TZD treatment. Whole cell extracts were prepared from fully differentiated 3T3-L1 adipocytes that were untreated or treated with 100 units/ml IFN γ or 2.5 μ M TZD. Proteasome activity was inhibited with epoxomicin (100 nM), lactacystin (5 μ M), or MG132 (10 μ M). Steady-state levels of PPAR γ were measured after 6 h. One hundred micrograms of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. The molecular mass of each protein is indicated to the left of the blot in kDa. The detection system was horseradish peroxidase-conjugated secondary antibodies (Sigma) and ECL (Pierce). This was a representative experiment independently performed three times.

RESULTS

Basal and IFN γ -mediated Targeting of PPAR γ to the Proteasome—We have previously shown that treatment of 3T3-L1 adipocytes with IFN γ leads to a decrease in the half-life of both PPAR γ proteins (13). Recent studies by Spiegelman and co-workers (34) have shown that TZDs target PPAR γ for proteasome-mediated degradation. These results suggest that targeting to the proteasome is an important regulatory event in the control of PPAR γ expression. Therefore, we examined PPAR γ expression in the presence of three distinct proteasome inhibitors. As shown in Fig. 1, treatment of 3T3-L1 adipocytes with either epoxomicin, lactacystin, or MG132 resulted in an increase in the levels of PPAR γ proteins under basal conditions or in the presence of IFN γ or TZD. Lactacystin and epoxomicin are highly specific proteasome inhibitors and confirm that the observed effects on degradation are due to proteasomal targeting (35, 36). As shown in Fig. 1, under steady-state conditions, IFN γ treatment of 3T3-L1 adipocytes leads to a substantial loss of PPAR γ when compared with control levels. The decrease in PPAR γ after IFN γ treatment is slightly greater than the decrease associated with the presence of synthetic ligand (TZD). Inhibition of the proteasome substantially reduces the IFN γ -induced decrease in PPAR γ expression. These results indicate that the loss of PPAR γ following IFN γ treatment is mediated by the targeting of PPAR γ to the 26 S proteasome. Interestingly, PPAR γ levels in both IFN γ - and TZD-treated adipocytes in the presence of proteasome inhibitors are less than the control levels under the same conditions. This result is consistent with studies that demonstrate that both IFN γ and TZDs can also down-regulate PPAR γ at the mRNA level (13, 37).

IFN γ -mediated Ubiquitin-PPAR γ Conjugation—Ubiquitin-proteasome-dependent degradation of a substrate requires two separate steps. First, the substrate is targeted to the proteasome via covalent tagging of the substrate with a polyubiquitin chain. The polyubiquitin-conjugated substrate is then recognized by the 26 S proteasome (22). These polyubiquitin-substrate conjugates are short lived, high molecular mass intermediates of the ubiquitin-proteasome pathway. Because IFN γ affects PPAR γ decay and this effect can be modulated by proteasome inhibitors, we hypothesized that there would be an increase in polyubiquitin-PPAR γ conjugates after IFN γ treatment. To test this theory, we examined the formation of endogenous PPAR γ -ubiquitin adducts in 3T3-L1 adipocytes. PPAR γ proteins were immunoprecipitated from whole cell extracts that had been incubated in the presence or absence of IFN γ for the times indicated in Fig. 2. The immunoprecipitations were analyzed by immunoblotting using either an anti-PPAR γ anti-

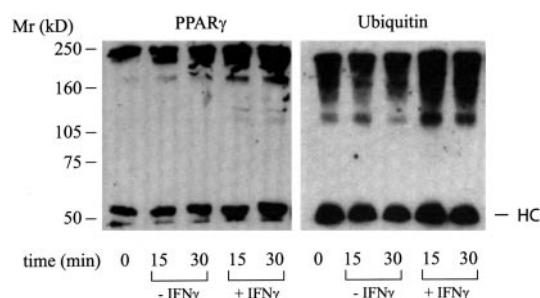


FIG. 2. IFN γ treatment is associated with an increase in PPAR γ -ubiquitin conjugates in 3T3-L1 adipocytes. Fully differentiated 3T3-L1 adipocytes were treated with IFN γ (100 units/ml) for 15 and 30 min after preincubation with MG132 (20 μ M) for 2 h. Control samples were incubated for the same time period without the addition of IFN γ . Whole cell extracts were harvested and immunoprecipitations were performed as described under "Experimental Procedures" using anti-PPAR γ . Western analysis was performed using either anti-PPAR γ (left) or anti-ubiquitin (right). HC represents IgG heavy chain.

body (Fig. 2A) or an anti-ubiquitin (Fig. 2B) antibody. As shown in Fig. 2, PPAR γ was detected in high molecular mass forms that are present under basal conditions and with increased intensity after IFN γ treatment. We also ectopically expressed octameric HA-tagged ubiquitin and PPAR γ 2 in NIH 3T3 cells and observed ubiquitin conjugation of PPAR γ under basal conditions and a significant increase in PPAR γ ubiquitin conjugation following IFN γ treatment (data not shown).

IFN γ -mediated Activation of PPAR γ —Based on our previous studies showing that IFN γ treatment of cultured adipocytes has the dual effect of suppressing PPAR γ transcription and increasing PPAR γ turnover (13), we hypothesized that IFN γ treatment may also decrease the transcriptional activity of PPAR γ . To test this prediction, we assayed the transcriptional activity of PPAR γ in NIH 3T3 cells using a luciferase reporter (DR1 luciferase) construct containing three PPAR γ response elements. This construct has previously been used to measure PPAR γ activity (34, 38). In this experiment, we also examined the effect of IFN γ on the transcriptional activity of the phosphorylation-deficient PPAR γ 2 S112A mutant. Numerous studies have shown that this mutant is more transcriptionally active and that phosphorylation at this site is associated with reduced PPAR γ activity (39–41). To measure PPAR γ activity, NIH 3T3 cells were transiently cotransfected with DR1 luciferase and PPAR γ 2 or PPAR γ 2 S112A in pSVSPORT vectors in the presence and absence of IFN γ or TZD. As shown in Fig. 3, IFN γ treatment activates PPAR γ 2 to the same extent as the ligand-dependent activation associated with TZD treatment. In

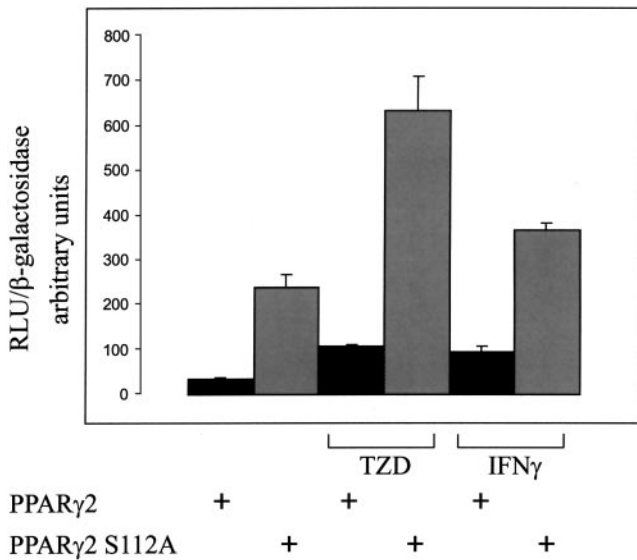


FIG. 3. IFN γ mediates transcriptional activation of both wild-type PPAR γ and PPAR γ S112A. NIH 3T3 cells were cotransfected with pDR1-luciferase and either wild-type PPAR γ or PPAR γ S112A. The cells were also transfected with pSV- β -galactosidase to correct for variability in transfection efficiency. After 24 h, the cells were incubated with IFN γ (100 units/ml) or darglitazone (TZD, 2.5 μ M) and harvested 6 h later. PPAR γ transcriptional activity was determined by calculating the ratio of luciferase activity (relative light units, RLU) to β -galactosidase activity. The experiment was independently performed in duplicate.

addition, activity of the PPAR γ 2 S112A was greater than wild-type PPAR γ 2, and the transcriptional activity of the mutant was also significantly induced by IFN γ treatment. However, the mutant was more potently activated by TZD treatment.

The Role of Ser¹¹² Phosphorylation in the Decay of PPAR γ —Because IFN γ and TZDs both activate PPAR γ and target it for degradation, we hypothesized that regulators of PPAR γ activation could also contribute to PPAR γ degradation. Therefore, we examined the contribution of PPAR γ Ser¹¹² phosphorylation on PPAR γ degradation because phosphorylation at this site has profound effects on PPAR γ activation. Fully differentiated 3T3-L1 adipocytes were pretreated with the MEK inhibitor, U0126, prior to the addition of IFN γ or a vehicle control. Turnover of PPAR γ was then measured in the presence or absence of cycloheximide. As shown in Fig. 4A, the turnover of both PPAR γ 1 and γ 2 was prolonged in the presence of the MEK inhibitor (*control + MEK I*). We also observed that inhibition of ERK1/2 activity abrogates the IFN γ -mediated decrease in the half-life of PPAR γ (Fig. 4A, *IFN γ + MEK I*). The results in Fig. 4A clearly demonstrate that the presence of the MEK inhibitor suppresses the decay of PPAR γ proteins in adipocytes under control and IFN γ -treated conditions. We also examined the effect of IFN γ and/or MEK I on PPAR γ levels in the absence of cycloheximide. The results in Fig. 4B confirm that ERKs 1 and 2 play a role in degradation of PPAR γ proteins under basal as well as IFN γ -mediated conditions. In Fig. 4, A and B, the expression of STAT 5A is shown as a loading control. The results in Fig. 4A also indicate that the decay of PPAR γ is much quicker than the decay of PPAR γ 2. Therefore, we performed an additional decay experiment to compare the decay of γ 1 and γ 2. Fully differentiated 3T3-L1 adipocytes were treated with cycloheximide, and whole cell extracts were isolated at various times over a 6 h period. Fig. 5A shows the decay of PPAR γ proteins under basal conditions. The γ 1 and γ 2 half-lives were calculated to be 58 min and 1.45 h, respectively. The *bottom panel* of Fig. 5A represents an enlarged display of four of the time points from the *top panel*. As shown in this panel, we were

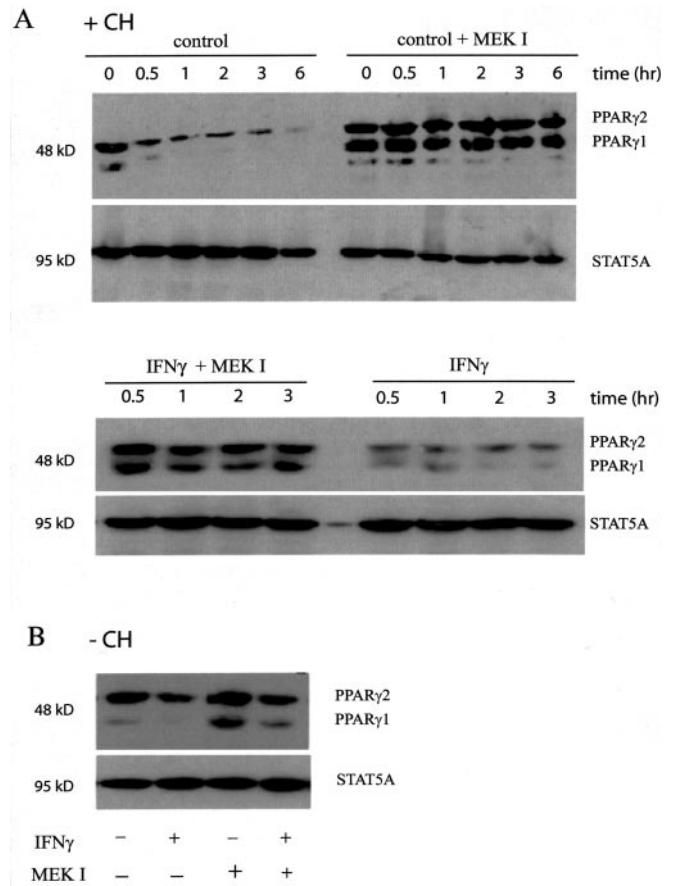


FIG. 4. Inhibition of ERK1/2 prolongs the half-life of PPAR γ proteins in adipocytes. PPAR γ expression was measured in the presence of 5 μ M cycloheximide (CH) (A) or under steady-state conditions (B) under control or IFN γ (100 units/ml)-treated conditions. Where indicated, the 3T3-L1 adipocytes were pretreated for 45 min with the MEK inhibitor (MEK I), U0126 (5 μ M). B, the cells were harvested after a 2-h incubation in the presence or absence of IFN γ . One hundred micrograms of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. Samples were processed, and results were visualized as described in the Fig. 1 legend. The molecular mass of each protein is indicated to the left of the blots in kilodaltons. This was a representative experiment independently performed three times.

also able to resolve the two bands of PPAR γ 1, which represent the Ser¹¹²-phosphorylated (*upper band*) and unphosphorylated forms of the protein. The decay experiment in Fig. 5A clearly demonstrates that PPAR γ 1 is more labile than γ 2. In addition, the unphosphorylated γ 1 disappears quicker than the phosphorylated form of γ 1. This pattern was also observed in the presence of IFN γ . (Fig. 5B).

Cellular Location of PPAR γ Degradation—The majority of PPAR γ proteins are found in the nucleus, and this raises the possibility that the nuclear, rather than cytosolic, ubiquitin-proteasome components may mediate the degradation of PPAR γ . To address this question, we treated 3T3-L1 adipocytes with IFN γ alone or in the presence of either MG132 or leptomycin B (Fig. 6). LMB acts as an irreversible inhibitor of the CRM-1-dependent nuclear export pathway via the modification of Cys⁵²⁹ of CRM-1 (33) and has been used to determine whether nuclear export is required for the degradation of nuclear proteins (42–44). We examined the decay of PPAR γ proteins following IFN γ treatment in the presence of either MG132 or LMB. The results in Fig. 6 indicate that MG132 prolongs the half-life of PPAR γ proteins, and the presence of LMB has no effect on PPAR γ decay. To confirm LMB activity, we assayed the cellular location of Mdm2 in 3T3-L1 adipocytes

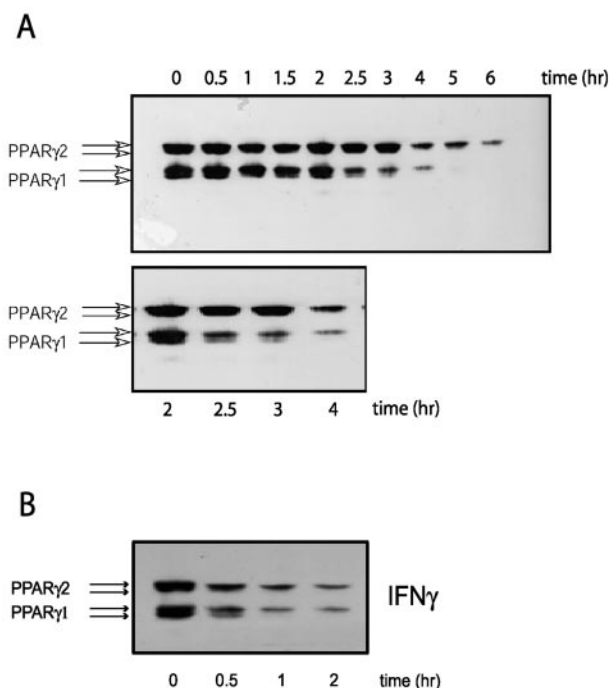


FIG. 5. PPAR γ 1 is more labile than PPAR γ 2, and the higher mobility form of PPAR γ 1 decays after the lower mobility form. A, whole cell extracts were prepared from fully differentiated 3T3-L1 adipocytes after incubation in the presence of 5 μ M cycloheximide for the indicated time points. Incubations were carried out in the absence (A) or presence (B) of IFN γ (100 units/ml). The lower panel (A) is an enlargement of the indicated time points from the upper panel. One hundred micrograms of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. Samples were processed, and results were visualized as described in the Fig. 1 legend. This was a representative experiment independently performed three times.

in the absence or presence of LMB. Mdm2 has been characterized as an ubiquitin ligase (E3) that shuttles between the nucleus and cytoplasm and is required for the degradation of p53 (22). Although p53 expression is down-regulated during differentiation of 3T3-L1 adipocytes, Mdm2 expression is maintained in fully differentiated 3T3-L1 adipocytes (45). Fig. 6B demonstrates that Mdm2 accumulates in the nucleus in the presence of LMB, indicating the effectiveness of LMB in these experiments. These results demonstrate that CRM-1-dependent nuclear export is not required for the degradation of PPAR γ following IFN γ treatment and strongly suggests that PPAR γ is degraded in the nucleus.

DISCUSSION

The novel observations in this study include the increased ubiquitin conjugation of PPAR γ following IFN γ treatment, the activation of PPAR γ transcriptional activity by IFN γ , evidence that PPAR γ 1 is substantially more labile than PPAR γ 2, evidence that serine phosphorylation of PPAR γ contributes to the turnover of PPAR γ proteins in adipocytes, and evidence that PPAR γ proteins are degraded by the nuclear ubiquitin-proteasome system. These results and recent findings by Spiegelman and co-workers (34) indicate that ubiquitin-proteasome-mediated degradation of PPAR γ is an important contributor to the cellular levels of PPAR γ proteins. Moreover, the cellular levels of PPAR γ appear to be important because transgenic mice that express half the normal amount of PPAR γ have been shown to be more insulin sensitive (12).

In light of our current findings and the studies cited above (13, 34, 39–41), we have formulated a model for the degradation of PPAR γ proteins in adipocytes. This model, illustrated in

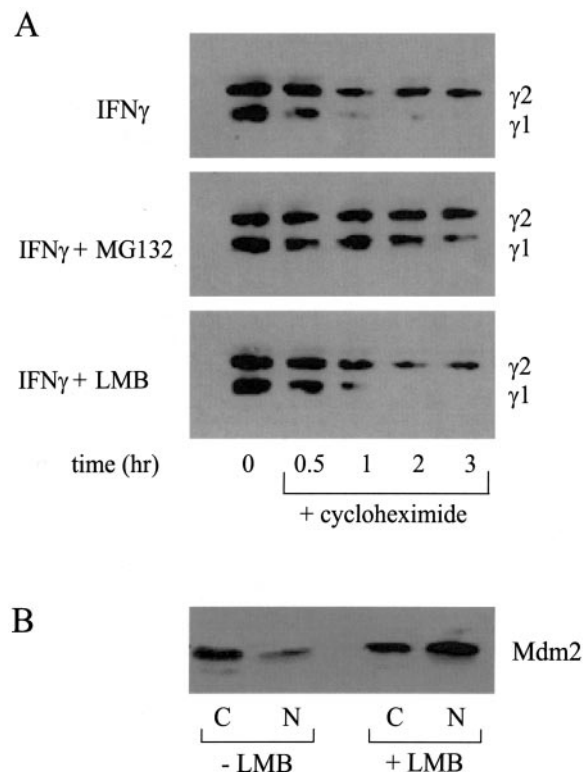


FIG. 6. PPAR γ degradation in adipocytes does not depend on nuclear export. A, fully differentiated 3T3-L1 adipocytes were incubated in the presence of cycloheximide (5 μ M) and harvested at the indicated time points. The adipocytes were treated with IFN γ alone or in the presence of MG132 (20 μ M) or leptomycin B (10 nM) as indicated. B, fully differentiated 3T3-L1 adipocytes were harvested after a 4-h incubation in the presence of ethanol (–LMB) or leptomycin B (+LMB, 10 nM). Cytosolic and nuclear extracts were obtained as described under “Experimental Procedures.” One hundred micrograms of each extract were separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. Samples were processed, and results were visualized as described in the Fig. 1 legend. This was a representative experiment independently performed two times.

Fig. 7, suggests that activation of PPAR γ by IFN γ , TZDs, or endogenous ligands is followed by ubiquitin-proteasome-mediated degradation. This model also suggests that serine phosphorylation contributes to PPAR γ degradation. The validity of this model is addressed in the following paragraphs.

Our results demonstrate that both PPAR γ 1 and PPAR γ 2 are targeted to proteasome under basal conditions and following IFN γ treatment of adipocytes. We have also observed ubiquitin conjugation of PPAR γ under basal conditions and demonstrated a substantial increase in ubiquitin conjugation of PPAR γ after IFN γ exposure. The increase in PPAR γ -ubiquitin conjugates occurred within 15 min of IFN γ treatment and precedes the decrease in PPAR γ observed in experiments measuring PPAR γ degradation. Our results demonstrating that proteasome inhibitors reduce the effect of IFN γ on PPAR γ expression and the results demonstrating the appearance of PPAR γ -polyubiquitin conjugates indicate that IFN γ treatment in adipocytes results in the rapid degradation of PPAR γ via the ubiquitin-proteasome pathway.

The rapid reduction in PPAR γ mRNA and protein levels following IFN γ treatment (13) led us to predict that IFN γ treatment would suppress PPAR γ activity in adipocytes. Surprisingly, IFN γ treatment of 3T3-L1 adipocytes was associated with the transcriptional activation of PPAR γ 2. Although unexpected, this result is consistent with the idea that nuclear hormone receptor turnover occurs concomitantly with transcriptional activation of these transcription factors (24). Ligand-

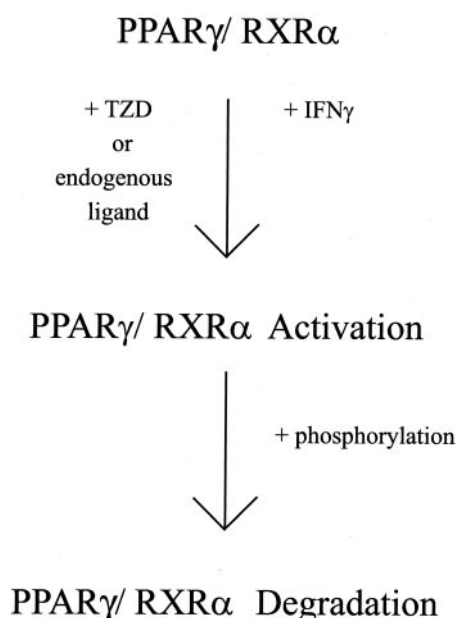


FIG. 7. **Proposed model for degradation of PPAR γ .** PPAR γ activation is mediated by ligand binding or exposure to IFN γ . Phosphorylation of PPAR γ influences the IFN γ and ligand-dependent degradation of PPAR γ .

dependent activation and subsequent degradation has been demonstrated for several other nuclear hormone receptors (26–30), and the paradigm of activation followed by ubiquitin-proteasome-dependent degradation has been extended to proteins such as protein kinase C (46). Although IFN γ has not been shown to be a ligand for PPAR γ , the activation of PPAR γ is a ligand-dependent process (47), and a recent study has demonstrated that PPAR γ 2 degradation is associated with the TZD-induced activation of PPAR γ 2 (34). Our data demonstrating that IFN γ treatment results in both the activation of PPAR γ 2 and the ubiquitin-proteasome-mediated degradation of PPAR γ suggest that IFN γ -mediated signaling in adipocytes may be associated with the binding of an endogenous ligand and the activation and subsequent degradation of PPAR γ . Moreover, IFN γ -induced PPAR γ 2 transcriptional activation is enhanced in the phosphorylation-deficient S112A mutant of PPAR γ 2. This result is consistent with previous findings showing that the mutation of Ser¹¹² to alanine in PPAR γ (Ser⁸² in PPAR γ 1) is associated with increased transcriptional activity (39, 40, 48).

The phosphorylation of PPAR γ by MAPKs has been described in various studies (39–41, 48, 49). Although neither IFN γ nor TZDs directly activate ERKs 1 and 2 in adipocytes, we found that inhibition of these MAPKs resulted in an inhibition of PPAR γ decay. Therefore, the mechanism(s) by which MAPKs influence PPAR γ degradation is not clear. However, phosphorylation plays an important role in targeting many substrates for ubiquitination and can either inhibit or increase the targeting of substrates to the ubiquitin-proteasome system (22, 23). In our experiments, we observed that both PPAR γ 1 and PPAR γ 2 migrate as a doublet on gels that have been run for 24–30 h (refer to Fig. 5). This doublet is easily distinguishable for PPAR γ 1. We confirmed that the slower migrating form corresponds to serine-phosphorylated PPAR γ 1, and the faster migrating form represents the unphosphorylated PPAR γ 1 proteins (data not shown), as has been previously described (34). The results in Fig. 5 demonstrate that the faster migrating form of PPAR γ 1 disappears prior to the phosphorylated form of the protein. The observed difference in the decay of these two forms of PPAR γ 1 suggest that phosphorylation of PPAR γ proteins may serve as a ubiquitin-proteasome targeting signal in

which PPAR γ is converted to the phosphorylated form prior to degradation by the ubiquitin-proteasome pathway. This hypothesis is also consistent with the increased activation of the S112A mutant, and we predict that the ubiquitin-conjugating machinery may not recognize the phosphorylation-deficient PPAR γ as well as the wild-type protein. We hypothesize that this may contribute to the increased activation observed with the S112A mutant. This model is also supported by our data demonstrating that inhibition of PPAR γ serine phosphorylation with the MEK inhibitor prolongs the half-life of PPAR γ proteins. All of these results support the hypothesis that serine phosphorylation of PPAR γ may influence its targeting to the ubiquitin-proteasome system. However, recent work from the Spiegelman laboratory (34) has shown that both the wild-type and the S112A form of PPAR γ 2 are degraded after ligand activation, but they did not determine whether the half-lives of these forms of the protein were different. Nonetheless, because the phosphorylation-deficient mutant can be degraded, it seems unlikely that serine phosphorylation is the only means by which PPAR γ proteins are targeted to the ubiquitin-proteasome system. Interestingly, the MAPK-regulated serine phosphorylation of the progesterone receptor has been shown act as a targeting signal for the degradation of this protein (28, 50).

We also investigated the cellular location of the IFN γ -mediated ubiquitin-proteasome-dependent degradation of PPAR γ . PPAR γ proteins are predominantly localized in the nucleus, and recent studies have demonstrated that the nuclear ubiquitin-proteasome is active in the degradation of selected substrates (42, 51, 52). Our results demonstrate that the IFN γ -mediated degradation of PPAR γ does not require CRM1-dependent nuclear export, indicating that IFN γ -induced PPAR γ degradation likely occurs in the nucleus. In the absence of serum deprivation, we observe active ERKs 1 and 2 in the nucleus of 3T3-L1 adipocytes (data not shown) and hypothesize that the presence of these kinases influences the nuclear decay of PPAR γ proteins. Finally, the observation that PPAR γ 1 is substantially more labile than PPAR γ 2 suggests that recognition of PPAR γ proteins by the ubiquitin-proteasome system in adipocytes is influenced by the 30-amino acid N-terminal extension found in PPAR γ 2. However, examination of the N-terminal residues of both forms of PPAR γ reveals that neither region contains the characteristic residues involved in the N-end rule targeting to the ubiquitin-proteasome system (53). Moreover, neither form contains a lysine residue necessary for ubiquitin conjugation (22). However, this study does not address the mechanisms underlying the differences in the half-lives of PPAR γ 1 and PPAR γ 2.

Recent studies (12, 54) have shown that reduced PPAR γ expression in mice (PPAR $\gamma^{+/-}$) is associated with resistance to weight gain along with protection from the insulin resistance that typically accompanies weight gain. In addition, genetic evidence indicates that decreased PPAR γ activity may protect against insulin resistance in humans (55). Conversely, PPAR γ is required for the formation of fat cells, and a lack of adipose cells is associated with insulin resistance and hyperglycemia (56). These studies suggest that a careful balance between PPAR γ expression and activity levels must be maintained to avoid development of diseases such as type II diabetes and obesity. The current study, along with a previous study showing that ligand activation of PPAR γ leads to ubiquitin-proteasome-dependent degradation of PPAR γ (34), suggests that the ubiquitin-proteasome pathway plays an important role in the regulation of PPAR γ expression in adipocytes.

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